AD_____

AWARD NUMBER: DAMD17-02-1-0673

TITLE: Acute Lung Injury Following Smoke Inhalation: Predictive Value of Sputum Biomarkers and Time Course of Lung Inflammation

PRINCIPAL INVESTIGATOR: Jefferey L. Burgess, M.D.

CONTRACTING ORGANIZATION: University of Arizona
                                            Tucson, Arizona 85722-3308

REPORT DATE: April 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                        Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                          Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

1. REPORT DATE *(DD-MM-YYYY)* April 2006	2. REPORT TYPE Annual	3. DATES COVERED *(From - To)* 15 Apr 05 – 14 Apr 06

4. TITLE AND SUBTITLE

Acute Lung Injury Following Smoke Inhalation: Predictive Value of Sputum Biomarkers and Time Course of Lung Inflammation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0673

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jefferey L. Burgess, M.D.

E-mail: jburgess@u.arizona.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Arizona
Tucson, Arizona 85722-3308

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Background: The role of lung inflammatory mediators in the development of lung injury following smoke inhalation is unknown. Objectives: To evaluate the predictive value and role of inflammatory mediators in acute lung injury following smoke inhalation. Specific aims: 1) Determine the predictive value of initial inflammatory markers in bronchial secretions of smoke inhalation victims for subsequent lung injury. 2) Measure longitudinal changes in inflammatory mediators in smoke inhalation victims. Study design: Bronchial secretions from 200-250 intubated patients with smoke inhalation injury will be evaluated for initial and longitudinal changes concentrations of substance P, TNF-α, IL-1, IL-8, and IL-10, as well as cell count and differential every two hours to a maximum of 72 hours. Initial lung inflammation and changes in inflammatory markers will be compared in patients without and without subsequent significant lung injury. Progress to date: We have enrolled 95 subjects to date and analyzed 23-32 subjects with for TNF-α, IL-1, IL-8, IL-10, sFASl, substance P, IL-1RA, α2M, MMP-9, and TIMP-1 concentrations. We have found temporal changes in IL-8 and IL-1β, and a significant relation between low initial IL-8 and risk of ALI. Further analysis of the concentrations of biomarkers predictive of the severity of subsequent lung injury await completion of recruitment.

15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)

smoke inhalation, acute lung injury, inflammation, cytokines

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	51	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION

The goal of this research is to identify inflammatory mediators playing key roles in acute lung injury (ALI) following smoke exposure. Our objectives are to determine the value of initial concentrations of these mediators in predicting later development of ALI, and to determine how the mediator concentrations change over time, which may also have predictive value and improve our understanding of the mechanism of smoke injury. We hypothesize that smoke inhalation results in rapid changes (within two hours) in lung inflammatory mediators, initial changes in lung inflammatory mediators are predictive of the extent of subsequent lung injury, and changes over time in lung inflammatory mediators will precede clinical findings of acute lung injury. Over the remaining years of this grant, we will be evaluating initial concentrations and changes over time of inflammatory mediators in pulmonary secretions of approximately 100 ventilated patients with smoke inhalation. The clinical course of these patients will be tracked, including % body surface area burn, days on a ventilator, days in ICU, pulmonary infiltrates, white blood cell count, fever, sputum volume, oxygen requirements, blood oxygenation, and development of ALI.

There were no adverse events or complaints received in the past year.

BODY

The two main specific aims of the study are: 1) Determine the predictive value of initial inflammatory markers in bronchial secretions of smoke inhalation victims for subsequent extent of lung injury; and 2) Measure longitudinal changes in bronchial inflammatory mediators in smoke inhalation victims. The specific aims have been divided into five tasks as shown in the approved Statement of Work timetable (with the task description modified to clarify the meaning of each step).

	Year 1	Year 2	Year 3	Year 4
Recruitment/Enrollment	→→→→→→→→→→→→→→→→			
Tracheobronchial fluid sample collection	→→→→→→→→→→→→→→→→			
Medical outcome data collection	→→→→→→→→→→→→→→→→			
Sample Analysis	→→→→→→→→→→→→→→			
Data analysis/Manuscript preparation	→→→→→→→→→→→→→→			

The major activity of the first year of this research was to obtain Institutional Review Board (IRB) approval from the Army, the University of Arizona, and the Maricopa Integrated Health System (MIHS), which is the parent institution of the Arizona Burn Center where the subjects are enrolled in the study and the bronchial suction material and clinical outcome data collected. This process took much longer than anticipated and therefore required shifting the start of all of the timetable tasks into year 2. We therefore continued subject recruitment and sample collection through year 4. In the past year, we have consented 26 additional subjects, bringing the total number of subjects to 95. Of these, 79% are male, and 21% female. The mean age of the subjects is 38.2 (±18.4) years. No ethnicity data was collected on these subjects.

Clinical outcome data has been collected on 90 of the 95 consented subjects. Data entry and cleaning for those subjects consented in the past 9 months is ongoing. Among these patients, there have been 10 deaths. Organ failure was recorded in 50 patients.

To date, arterial blood gas data, including PaO_2, FIO_2, and positive end respiratory pressures (PEEP), were collected on 83 subjects, generally starting within 6 hrs of smoke exposure and continuing for 72 hrs post-intubation.

Tracheobronchial samples were collected at approximately two hour intervals, starting within 6 hours of intubation for over 90% of subjects. Laboratory assays on the tracheobronchial samples have required considerable experimentation with different preservation techniques and different types of assays. To date, we have collected and processed over 1200 samples on a total of 90 subjects, and incorporated time of sample collection and laboratory results for 28 patients into our database.

For 32 subjects, we have completed assays of Interleukin-1 beta, Interleukin-8, and Tumor Necrosis Factor-alpha (IL-1β, IL-8 and TNF-α, respectively) using the R&D Systems Quanti-Glo Elisa Kits. High, medium and low controls were added to the testing protocol for all assays after 11/15/04 to ensure accuracy of the testing protocol. Both soluble Fas Ligand (sFASL) and Transforming Growth Factor-beta 1 (TGF-β1) were measured on 23 subjects using the R&D Systems Quantikine Elisa Kits, after verification of the testing procedure. Measurement of protein concentration using the Sigma BCA-1 Protein Determination Reagent Kit was completed after kit verification for use on sputum and bronchial lavage samples. Urea nitrogen was performed using the Pointe Scientific, Inc., Reagent Set # B7550-400 after the methodology was verified using the protocol for verification of new tests. Urea levels in the tracheobronchial secretions were consistently low. In the past year, additional biomarkers have been assayed on 23 subjects with tracheobronchial samples within the first six hours and arterial blood gas measures. Interleukin-1 Receptor Antagonist (IL-1 RA), tissue inhibitor of metalloproteinase-1 (TIMP-1), and matrix metalloproteinase-9 MMP-9 were measured using R&D Systems Quantikine Kits, and alpha-2 macroglobulin (α2M) was measured using ALPCO Diagnostics Kits with in-house controls.

Cell counts and differentials were completed on 83 subjects. The initial procedure called for preservation of the sample in methanol. Because of the unavoidable time delay between collection and processing, methanol did not preserve the cells sufficiently for accuracy for either the cells counts or the differentials. 40% Glycerol was used with better cell counting results, but cells were too degenerated upon storage to accurately perform the differential. Cytolyte was then used yielding cells that displayed less cellular disintegration; however, the cells contracted, which made cell counting difficult and the staining characteristics for the differential were unreliable. Histochoice was then used which showed much better results for cell counting and differentiation and is the method of preservation used at this time.

Substance-P was assayed on 16 subjects using the R&D Elisa Assay Kit. Results were consistently under the detection limit. After conferring with company representatives and laboratorians experienced in testing Substance-P levels, an additional preliminary concentration/purification protocol using a C-18 reverse phase cartridge (Sep-Pak) was

performed on several subjects. Repeated Substance-P analyses remained under the detection limits in our tracheobronchial samples, so these assays were discontinued.

Data analysis on the first three years of data was submitted for publication. We have found that PaO_2/FIO_2 decreases over time in these patients ($p<0.0001$), generally reaching its nadir at about 20-30 hours post-intubation (Figure 1). Almost 66% of patients developed PaO_2/FIO_2 <200 within 72 hrs of exposure (Table 1), and 35% of a subgroup of 23 with tracheobronchial biomarkers measured within 6 hrs of intubation developed ARDS (Table 2). In random coefficients modeling, IL-1β and IL-8 increased significantly in the first six hours post-exposure ($p<0.001$) (Fig 2A), but no significant temporal trends in IL-10, TNF-α, TGF-β, sFasL or C5a were found (Fig 2B). Initial IL-8 concentrations were significantly lower in subjects who had a minimum PaO_2/FIO_2 of ≤ 200 with PEEP <6, than in subjects whose minimum ratio was >200 ($p=0.03$).

Table 1: Characteristics of subjects (enrolled June 2003-January 2005)

Mean age (range)	39.2 yrs (1.8 - 88)
Male	51/61 (83.6%)
Total body surface burn	
<15%	23/61 (37.7%)
15-35%	17/61 (27.9%)
>35%	21/61 (34.4%)
Bronchial severity	
1	14/51 (27.5%)
2	16/51 (31.4%)
3	11/51 (21.6%)
4	7/51 (13.7%)
5	3/51 (5.9%)
Organ failure	31/60 (51.7%)
Chest infiltrates*	13/23 (56.5%)
Trauma	9/60 (15.0%)
Fracture	6/60 (10.0%)
$PaO_2/FIO_2 \leq 200$ at ≤ 72 hrs	38/58 (65.5%)
Died within 72 hrs	3/61 (4.9%)

* Only evaluated in subjects with measured tracheobronchial mediator concentrations collected within 6 hours of intubation.

Table 2. Comparison of burn subject characteristics by different outcome measures within 72 hrs of exposure: PaO2/FIO2 ≤ 200 v. > 200; PaO2/FIO2 ≤ 200 v. > 200 restricted to PEEP < 6; and presence v. absence of ARDS (bilateral infiltrates and PaO2/FIO2 ≤ 200).

N (%)	PaO_2/FIO_2 ≤ 200 (n=38)	PaO_2/FIO_2 > 200 (n=20)	PaO_2/FIO_2 ≤ 200 & PEEP<6 (n=21)	PaO_2/FIO_2 > 200& PEEP<6 (n=20)	ARDS (n=8)	not ARDS (n=15)
Males	34 (89.5)	15 (75)	19 (90.5)	14 (70)	8 (100)	14 (93.3)
Females	4 (10.5)	5 (25)	2 (9.5)	6 (30)	0	1 (6.7)
Total burn						
<15%	11 (28.9)	11 (55)	8 (38.1)	10 (50)	0	8 (53.3)
15-35%	10 (26.3)	6 (30)	4 (19.0)	7 (35)	1 (12.5)	3 (20.0)
>35%	17 (44.7)	3 (15)*	9 (42.9)	3 (15)	7 (87.5)	4 (26.7)**
Organ failure	20 (54.1)	9 (45)	9 (45)	7 (35)	4 (50)	5 (33.3)
Trauma	6 (16.2)	3 (15)	4 (50)	1 (5)	3 (37.5)	2 (13.3)
Fracture	4 (10.8)	2 (10)	2 (10)	1 (5)	2 (25)	1 (6.7)

Fisher's Exact test, * p=0.048, ** p=0.011

Figure 1. PaO$_2$/FIO$_2$ ratios are inversely related to time since exposure (p=0.001 in a random coefficients model).
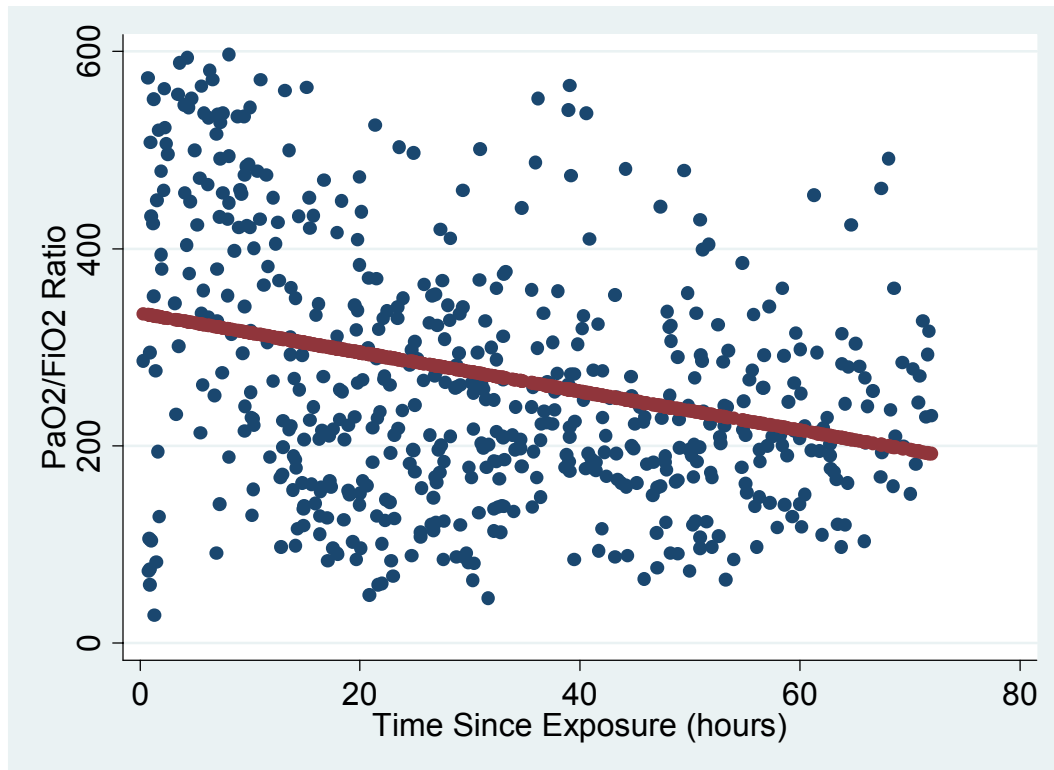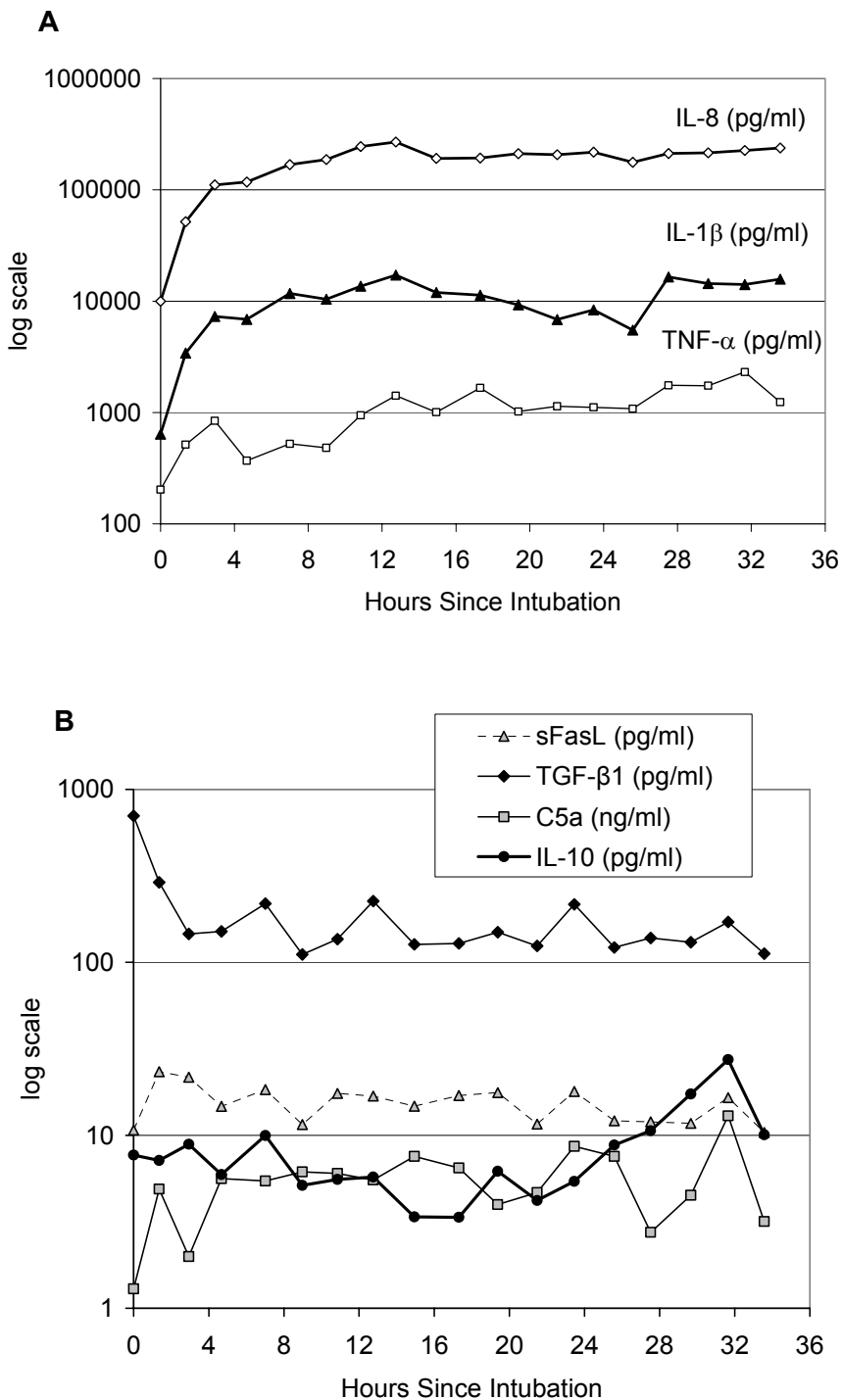
Figure 2. Mean cytokine levels in tracheobronchial fluid samples over time since intubation. A) Mean levels of IL-8, IL-1β, and TNF-α.   B) Mean levels of TGF-β1, sFasL, IL-10 and C5a.

KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated that in our patient population smoke inhalation victims almost uniformly manifest a decline in their PaO_2/FIO_2 ratio to below 300, which is consistent with development of acute lung injury.
- We have demonstrated that bronchial suction material can be used for longitudinal analysis of cytokines using commercially available ELISA kits.
- We have found that IL-1β and IL-8 increased significantly in the first six hours post-exposure, and that initial IL-8 concentrations were significantly lower in patients with minimum PaO_2/FIO_2 ratios greater than 200.
- We have begun to look at additional biomarkers, including MMP-9, TIMP-1, IL-1 RA, and α-2M.

REPORTABLE OUTCOMES

Two poster presentations at scientific meetings describing our preliminary findings:

1) *American Thoracic Society 100th International Conference*, Orlando, FL   May 25, 2004

"Longitudinal changes in tracheobronchal suction fluid inflammatory mediators following smoke inhalation"

2) *American Thoracic Society 101st International Conference*, San Diego, CA   May 23, 2005

"Use of tracheobronchial suctionate inflammatory markers to predict subsequent lung injuring in smoke inhalation victims"

3) Journal article submitted to: *The American Journal of Respiratory and Critical Care Medicine*:

"Tracheobronchial markers of lung injury in smoke inhalation victims"

Jefferey L. Burgess, MD, MPH; Kevin Foster, MD; Margaret Kurzius-Spencer, MS, MPH Karen J. Richey, RN; Duane Sherrill, PhD; Sally Littau, BS; Arun B. Josyula, MD, MPH; Richard B. Goodman, MD; Scott Boitano, PhD

CONCLUSIONS

Smoke inhalation injury continues to cause significant morbidity and even mortality, as demonstrated by the clinical outcomes of our subjects to date. No diagnostic test or specific pharmaceutical therapy is available for acute lung injury following smoke exposure. We have shown that longitudinal evaluation of tracheobronchial suctionate from smoke inhalation victims can be analyzed for measurement of inflammatory mediators. If we can show that specific inflammatory mediators secreted in the lungs in the first two-six hours following smoke exposure are predictive of later decline in PaO_2/FIO_2 ratio, then it will be reasonable to consider evaluation in animal models and, if successful, in human clinical trials, of

pharmacological agents working through antagonism or promotion of the effects of these mediators.

Future directions include the analysis of additional inflammatory mediators and measurement of these inflammatory mediators in small animal models of smoke exposure. Thus far, we have found a rapid increase in tracheobronchial fluid of both IL-1β and IL-8 in the first six hours post-intubation and an increase in percent neutrophils within 12 hours of smoke exposure. Contrary to expectation, we found that initial IL-8 concentrations were lower in subjects who later developed more severe lung injury, and a similar trend was found with IL-1β. A possible explanation may be that in patients with severe smoke exposure, there are modifications to or loss of IL-8. In the next year, we plan to analyze the tracheobronchial concentration of alpha-2-macroglobulint (α2M), which is a measure of altered lung permeability. This will provide an additional intermediate endpoint which can be related both to initial cytokine concentrations as well as clinical endpoints.

In addition, we plan to analyze tracheobronchial concentrations of IL-1RA, MMP-9, and TIMP-1 in our subjects, which are additional inflammatory mediators that may be predictive of the development of ALI and also could serve as therapeutic targets. As mentioned in previous reports, the selection of additional inflammatory mediators will be based in part on the availability of potential therapeutic interventions associated with the selected mediators.

REFERENCES

Please see Appendix 3 for documentation of literature review. There have been no recently published articles that would alter our protocols.

APPENDICES

1) Abstract, *American Thoracic Society 100th International Conference*   May 25, 2004
   Orlando, FL
   "Longitudinal changes in tracheobronchal suction fluid inflammatory mediators following smoke inhalation"

2) Abstract, *American Thoracic Society 101st International Conference*   May 23, 2005
   San Diego, CA
   "Use of tracheobronchial suctionate inflammatory markers to predict subsequent lung injuring in smoke inhalation victims"

3) Paper submitted to: *American Journal of Respiratory and Critical Care Medicine*

   "Tracheobronchial markers of lung injury in smoke inhalation victims"

   Jefferey L. Burgess, MD, MPH; Kevin Foster, MD; Margaret Kurzius-Spencer, MS, MPH Karen J. Richey, RN; Duane Sherrill, PhD; Sally Littau, BS; Arun B. Josyula, MD, MPH; Richard B. Goodman, MD; Scott Boitano, PhD.

4) REQUIRED DOCUMENTS

   Sample Collection Protocol

   Current verification of Human Subject Training

   IRB Periodic Review--Approval of Study Continuation through April 15, 2006

   Continuing Review Application Letter

Appendix 1.

[C52] [Poster: E14] Longitudinal Changes in Tracheobronchal Suction Fluid Inflammatory Mediators Following Smoke Inhalation

J.L. Burgess, A. Josyula, K.N. Foster, T.A. Hysong, N.S. Francis University of Arizona, Tucson, AZ; Maricopa Integrated Health System, Phoenix, AZ

Rationale: The role of inflammatory mediators in the development of human smoke inhalation injury is not well understood. We hypothesize that initial changes in lung inflammatory mediators are predictive of the extent of subsequent lung injury. *Methods*: As a first step in investigating this process, mediator concentrations in tracheobronchal secretions of ventilated patients were collected every two hours over the first 72 hours following smoke inhalation. Sample supernatants were analyzed by ELISA. *Results*: For the first four subjects for which samples have been analyzed, comparing the initial mediator concentration with the peak level gave the following fold increases: interleukin (IL)-1β 70-106; IL-8 6-115; IL-10 1-7; TNF-α 225-2560; and substance P 1-4. The longitudinal changes in one of the subjects enrolled in the study are illustrated in Figure 1. *Conclusions*: Longitudinal collection of tracheobronchal suction material provides a means of measuring changes in inflammatory mediators which will be evaluated for association with development of acute lung injury.

Figure 1. Longitudinal changes in inflammatory mediator concentrations in a single subject
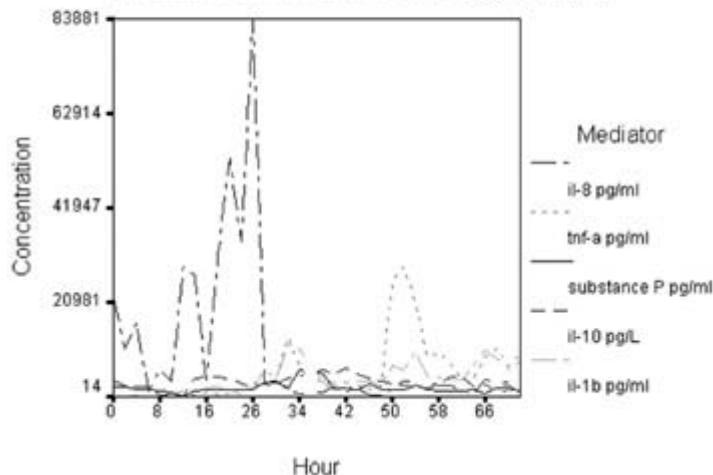
Tuesday, May 25, 2004 8:15 AM

Appendix 2.

**[**] Thematic Poster Session (Abstract Page: A641) Session: 8:15 am-4:15 pm,
ENVIRONMENTAL AND OCCUPATIONAL PULMONARY TOXICOLOGY**

 American Thoracic Society : Abstract # 956596

*Title***: Use of tracheobronchial suctionate inflammatory markers to predict subsequent lung injury in smoke inhalation victims.**

J.L. Burgess, MD, MPH [1], K.N. Foster, MD [2], S.R. Littau [1], M. Kurzius-Spencer, MS, MPH [1], K.J. Richey, RN [2], A.B. Josyula, MD, MPH [1] and R.M. Shipitalo [1].
[1] University of Arizona, Tucson, AZ
[2] Arizona Burn Center, Maricopa Integrated Health System, Phoenix, AZ.

Rationale: Smoke inhalation victims are at high risk of developing acute respiratory distress syndrome (ARDS). Given the delay of 12 or more hours from exposure to development of ARDS, a prognostic test applied early in the clinical course could potentially identify patients for whom specific interventions may be of value. *Methods*: Patients with inhalation injury admitted to a regional burn center and requiring intubation were eligible for the study. Tracheobronchial suction fluid was collected every two hours. Sample supernatants were analyzed for interleukin-1, -8, (IL-1, IL-8) and tumor necrosis factor alpha (TNF-) by ELISA. Medical history and clinical course including arterial oxygenation (PaO2) and fraction of inspired oxygen (FIO2) were collected. *Results*: Mean PaO2/FiO2 decreased over time, generally reaching its nadir at 18-28 hours post-intubation. Regression models were run to assess the relationship between early IL-1, IL-8 and TNF- concentrations and subsequent PaO2/FiO2 measurements, adjusting for potential confounders including age, asthma, COPD, percent of full thickness body surface burned, and fractures suffered. In an analysis of eight patients with complete information, the log of IL-1 from a bronchial sample at 4 hours post-intubation (p=.008), age (p=.023), and % body surface burned (p=.022) were all significant predictors of PaO2/FiO2 at 18 hours. At similar time points, tracheobronchial TNF-, but not IL-8, was predictive of later PaO2/FiO2. *Conclusion*: In patients admitted to a burn center with smoke inhalation requiring intubation, IL-1 concentrations in tracheobronchial suction material at four hours were predictive of PaO2/FIO2 at 18 hours after exposure.

Appendix 3.

TRACHEOBRONCHIAL MARKERS OF LUNG INJURY IN

SMOKE INHALATION VICTIMS

Jefferey L. Burgess, MD, MPH[1]; Kevin Foster, MD[2]; Margaret Kurzius-Spencer, MS, MPH[1]

Karen J. Richey, RN[2]; Duane Sherrill, PhD[3]; Sally Littau, BS[1]; Arun B. Josyula, MD, MPH[1];

Richard B. Goodman, MD[4]; Scott Boitano, PhD[3,5]

1. Environmental and Community Health, University of Arizona, Tucson, AZ

2. Arizona Burn Center, Maricopa Integrated Health System, Phoenix, AZ

3. Arizona Respiratory Center, University of Arizona, Tucson, AZ

4. University of Washington, Seattle, WA

5. Department of Physiology, University of Arizona, Tucson, AZ

Corresponding author: Jefferey L. Burgess, MD, MPH

Associate Professor, Environmental and Community Health

University of Arizona Mel and Enid Zuckerman College of Public Health

Mailing Address: 1435 N Fremont Ave, Tucson, AZ 85719. USA

Fax: (520) 882-5014; Telephone: (520) 882-5852 Ext 12; e-mail: jburgess@u.arizona.edu

Running head (max 35 characters): Markers of Smoke Inhalation Injury

MeSH Heading and Tree Number: Smoke Inhalation Injury C21.866.200.322.800

Word count (excluding abstract and references): 3,611

ABSTRACT

Rationale: Although smoke inhalation injury victims frequently develop acute respiratory distress syndrome (ARDS), no early prognostic tests are available. *Objectives:* To describe longitudinal changes in tracheobronchial fluid inflammatory markers and assess the value of initial concentrations as predictors of subsequent lung injury. *Methods*: Arterial oxygenation (PaO_2) and fraction of inspired oxygen (FIO_2) were recorded and tracheobronchial suction fluid was collected every two hours for 72 hours from intubated smoke inhalation injury victims admitted to a regional burn center. Suctionate was assayed for interleukins (IL-1β, -8, and -10), tumor necrosis factor-alpha (TNF-α), transforming growth factor beta (TGF-β), soluble Fas ligand (sFasL), and complement factor 5a (C5a). Temporal trends in biomarker concentrations and relationships between their initial concentrations and the lowest PaO_2/FIO_2 within 72 hours were assessed. *Measurements and Main Results:* Thirty-eight (66%) of 58 patients developed PaO_2/FIO_2 <200 within 72 hrs of exposure, and 8 (35%) of a subgroup of 23 with tracheobronchial biomarkers measured within 6 hrs of intubation developed ARDS. In random coefficients modeling, IL-1β and -8 increased significantly in the first six hours post-exposure (p<0.001), but no significant temporal trends in IL-10, TNF-α, TGF-β, sFasL or C5a were found. Initial IL-8 concentrations were significantly lower in subjects who had a minimum PaO_2/FIO_2 of ≤ 200 with PEEP <6, than in subjects whose minimum ratio was >200 (p=0.03). *Conclusions*: In smoke inhalation victims, tracheobronchial IL-1β and IL-8 increase rapidly, and low initial IL-8 may be a risk factor for severity of lung injury.

Keywords: Inhalation Burns, Acute Respiratory Distress Syndrome, Interleukin-8, Interleukin-1 beta.

INTRODUCTION

In the U.S., there are more than one million burn injuries annually (Brigham and McLoughlin 1996; Enkhbaatar and Traber, 2004). Respiratory complications are the major cause of mortality from smoke inhalation and burns, and early complications commonly include increased alveolar permeability to protein, acute pulmonary edema, and an accumulation of both pro- and anti-inflammatory cytokines (Laffon et al., 1999; Matthay and Zimmerman 2005; Park et al., 2001), all hallmarks of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). The general mortality rate associated with ALI/ARDS exceeds 40% (Martin et al., 2005), although it varies depending on the underlying cause.

Specific risk factors for ARDS due to smoke inhalation injury include duration and dose of smoke (Hales et al., 1997), age (Dancey et al., 1999), extent of skin burn (Enkhbaatar and Traber, 2004) and chronic lung and liver disease (Ware and Matthay, 2000). However, indices of oxygenation and ventilation and lung injury scores are not predictive of mortality risk at the time of diagnosis (Ware and Matthay, 2000). At present, there is no diagnostic test available that could be applied early in the clinical course to predict the extent of subsequent lung injury. In addition, no specific therapy is available for smoke inhalation injury other than supportive care.

There appear to be two main phases prior to the development of smoke or toxic chemical-induced ARDS: direct chemical injury to epithelial cells, and subsequent inflammatory response. Although the specific time course for these phases in humans is not thoroughly understood, measurable changes in lung lumenal fluids during epithelial compromise and inflammatory cell recruitment could lend to the development of prognostic and/or diagnostic

tests. Evaluation of changes in the lung through direct measure of mediators in the

tracheobronchial suctionate rather than in the blood is likely to provide the best measure of

lung inflammation (Suter et al., 1992). Release of inflammatory mediators by macrophages and

epithelial cells also increases the recruitment of neutrophils to the lung, neutrophil activation,

and their migration into the alveolar space. Such lung inflammation alters alveolar

permeability, with resultant entry of water and blood proteins into the alveoli and the

development of pathology associated with lung injury (Matthay and Zimmerman, 2005;

Enkhbaatar and Traber, 2004).

In this study, we focused on the initial 72 hours following exposure, when ALI and/or

ARDS could potentially be limited or prevented, and measured secreted inflammatory

mediators associated with smoke exposure, ARDS, or epithelial cell injury. Our hypothesis

was that early increases in proinflammatory cytokines would be predictive of the extent of lung

injury, as measured by the lowest PaO_2/FIO_2. Our study objectives were to measure

longitudinal changes in bronchial inflammatory mediators and to determine the predictive

value of initial inflammatory markers in bronchial secretions for subsequent extent of lung

injury.

METHODS

Subjects and Data Collection

This study was approved by the Institutional Review Boards of the University of Arizona, Tucson, AZ, and Maricopa Integrated Health System, Phoenix, AZ. Patients admitted to the Arizona Burn Center between June 2003 and April 2005 who had suffered smoke inhalation and required intubation participated in this study. Consent was obtained from family members of the patients. Respiratory technicians recorded partial pressure of arterial oxygen (PaO_2), fraction of inspired oxygen (FIO_2), and extent of positive end respiratory pressure (PEEP) at approximately two-hour intervals. The definition of ALI and ARDS used in this study followed the American-European consensus conference on ARDS: presence of acute hypoxemia with a PaO_2/FIO_2 ratio of 300 mmHg or less (for ALI) or of 200 mmHg or less (for ARDS); bilateral infiltrates seen on frontal chest radiograph, consistent with pulmonary edema; and no clinical evidence of left atrial hypertension (Rubenfeld et al, 2005). A radiologist blinded to the subjects' outcomes read chest radiographs for presence or absence of bilateral infiltrates consistent with ARDS. Organ failure was physician-diagnosed and included respiratory, renal, cardiopulmonary, and multiple organ failure. Fractures included spinal, hand, skull, face, and multiple fractures. Tracheobronchial injury was assessed through bronchoscopy, which was performed as soon as possible after admission. A scoring system of 1 to 5 was assigned based on the severity of carbon staining, edema, secretions, and erythema.

Tracheobronchial Suctionate Protocol

As part of routine pulmonary care, tracheobronchial suction fluid was collected at two-hour intervals for up to 72 hours. The respiratory technician instilled normal saline (15 ml in adult and 5 ml in pediatric patients) through the tracheobronchial tube, and then used a suction catheter with Lukens trap to aspirate as much fluid as possible. Samples of 0.5 ml of the sample and 0.5 ml of fixative (Histochoice MB, Amresco, Solon, OH) were mixed and refrigerated at 2-8 °C prior to cytocentrifugation and cell count. The remainder of the sample was frozen at -80 °C. After transporting the sample to the University of Arizona, the suctionate was thawed and the sample volume was combined with an equal volume of 0.1% dithiothreitol (DTT) solution (Sputolysin; Calbiochem, San Diego, CA). Samples were vortex mixed and then centrifuged at 2400 RPM for 16 minutes at 4 °C. The supernatant was frozen at -80 $^{\circ}$C until time of cytokine analysis.

Laboratory Procedures

Levels of interleukin (IL) -1 beta (IL-1β), IL-8, and tumor necrosis factor-alpha (TNF-α) were measured over the first 36 hours post-smoke exposure using R&D Systems Quanti-Glo Elisa and IL-10 using a R&D Systems High Sensitivity Quantikine kit. Soluble Fas ligand (sFasL) and transforming growth factor beta (TGF-β1) were measured using R&D Systems Quanti-Kine Elisa kits. Levels of Complement 5a (C5a) were measured using the BD Biosciences Human C5a ELISA Kit (BD Biosciences Pharmingen, San Diego, CA). Substance-P was assayed with and without preliminary concentration/purification protocol using a C-18 reverse phase cartridge (Sep-Pak) on 16 subjects using the R&D Elisa Assay Kit. Protein concentration was measured using the Sigma BCA-1 Protein Determination Reagent

Kit and measurement of urea nitrogen was performed using the Pointe Scientific, Inc., Reagent Set.

Statistical Analysis

Cytokine concentrations were either $\log_{10}$-transformed for parametric analyses or compared using non-parametric tests if greater than 10% of values were below the limit of detection. Fisher's exact test was used to assess associations between categorical variables, an exact binomial test was used to compare proportions, and the t-test and Mann-Whitney U test were used to compare initial mean or median levels of biomarkers by clinical outcome, respectively. Longitudinal/temporal relations between biomarkers and sample times and between PaO_2/FIO_2 ratio and sample times were evaluated using random coefficients models (Stata 9.1). Multiple linear regression models were run to determine whether initial cytokine levels could predict the lowest PaO_2/FIO_2 ratio recorded within 72 hours. In the first set of models, we adjusted for PEEP by including it as a categorical predictor variable (PEEP < 6, 6-10, >10 cm H_2O). The second set of models was run using the lowest PaO_2/FIO_2 ratio recorded with a PEEP setting of < 6 as the dependent variable. Exploratory cross-sectional analyses of cytokines and PaO_2/FIO_2 ratios at different time points were performed using Spearman Rank correlation analysis, and adjusted for multiple comparisons using a Bonferroni correction (Stata 9.1). Tests of significance were all two-sided and a critical value of p = 0.05 was used.

RESULTS

Study Subjects

Demographic, medical history and exposure data were collected on 61 subjects. Arterial

blood gas data were collected generally starting within 6 hrs of acute smoke exposure (0.33-20

hrs, median 3.3 hrs) and continuing up to 72 hrs post-intubation (Table 1). The study

population was primarily male and ranged from 2-88 yrs of age. Approximately one third of

subjects suffered total body surface area burns on more than 35% of their body. Including all

blood gas measurements up to 72 hours, there was a decrease of 1.64 in the ratio of PaO_2/FIO_2

with each cumulative hour since exposure after adjusting for PEEP (<6, 6-10, >10 cm H_2O)

setting (p<0.001, random coefficients model) (Figure 1). PEEP levels were not significant

predictors of PaO_2/FIO_2, and when dropped from the model, the decrease in the PaO_2/FIO_2

ratio was 1.97 with each hour increase in cumulative time. Restricting the analysis to only

ABGs with PEEP settings < 6, there was an hourly decrease of 2.55 in the PaO_2/FIO_2 ratio

(p=0.034). Nearly two-thirds had a PaO_2/FIO_2 ratio of 200 or less in the first 24 hours post-

exposure.

Three (5%) of the 61 subjects died within 72 hrs of exposure. The patients who died were

significantly older than those who did not (61.9 v. 38.0 yrs, p=0.03), and the proportion of

those who died with high scores (>3) on the bronchial severity scale was significantly higher

than those of survivors (p<0.002, exact binomial). There was no significant association

between concurrent trauma, fractures, sepsis, or chest infiltrates in patients who died compared

with survivors. All of the patients who died suffered full thickness plus partial thickness burns

covering more than 15% of their total body surface area, compared with 60% of patients who survived, although this difference was not statistically significant (p<0.158, exact binomial).

Tracheobronchial inflammatory mediators

Initial tracheobronchial samples were collected within three hours of exposure in 64% and within six hours of exposure in 88% of subjects. Log-transformed concentrations of IL-1β, IL-8, and TNF-α within subjects were highly correlated. IL-1β and -8 increased significantly from time from intubation (0-36 hours), but most steeply in the first six hours (both p< 0.001, random coefficient models) (Figure 2A). Log values of total protein showed a small but significant decline over 36 hours (p=0.002). No significant linear trends over cumulative time were observed for TNF-α (Figure 2A), IL-10, TGF-β1, C5a, or sFasL (Figure 2B)**.** C5a concentrations were assessed in only 10 subjects within the first 6 hours post-intubation due to limited suctionate volume. Substance P levels were consistently below the limit of detection, even after additional preliminary concentration and purification were performed. Due to initial methodological problems with cell lysis during storage, neutrophil counts were available for only 10 subjects within 6 hours of intubation. The percentage of neutrophils within the total cell count in the suctioned tracheobronchial fluid increased by 2.36% per hour in the first 12 hours post-intubation (random coefficients model, p=0.019), from approximately 60 to 88%.

     In Table 2, subject and injury characteristics are compared for three different dichotomous outcomes: 1) patients who had PaO_2/FIO_2 ratios $\leq$ 200 within 72 hours of intubation v. subjects whose ratios did not fall below 200; 2) a subgroup of patients who had PaO_2/FIO_2 ratios $\leq$ 200 v. greater than 200 with PEEP $\leq$6 cm H_2O; and 3) patients who met clinical criteria for ARDS (bilateral infiltrates on chest radiographs and PaO_2/FIO_2 below 200) v. those

who did not meet criteria for ARDS. The only statistically significant associations found were that percent total body surface area burns was higher in subjects with a minimum PaO_2/FIO_2 equal to or below 200 as compared with those with minimum PaO_2/FIO_2 above 200, and those with ARDS as compared to those without ARDS. Percent of subjects with fractures, trauma and organ failure did not differ significantly by outcome variable.

Study results restricted to subjects with measurement of tracheobronchal mediators within 6 hours is presented in Table 3. There were no significant differences in initial tracheobronchial marker levels, protein or urea, by minimum PaO_2/FIO_2 ratio (less than or equal to 200 v. greater than 200). In subjects with minimum PaO_2/FIO_2 ratios $\leq$ 200 and PEEP <6, the earliest measured concentrations of IL-8 were significantly lower (p=0.03) than in subjects with ratios >200, although this relationship was not found in the comparison of subjects developing and not developing ARDS. Subjects with ARDS had significantly lower initial levels of C5a (p=0.044) than those without, though this is based on a very small sample size.

In exploratory cross-sectional analyses of the relation of TGF-β production to PaO_2/FIO_2 ratios at subsequent time points, the concentration of TGF-β1 at three hours was positively correlated with PaO_2/FIO_2 at 16 hours (rho=0.689), at 7 hours was positively correlated with PaO_2/FIO_2 at 22 hours (rho=0.506), and at 7 and 11 hours was positively correlated with PaO_2/FIO_2 at 43 hours (rho=0.650 and 0.536, respectively). However, these findings were not hypothesis driven and none of these correlations were statistically significant after using a Bonferroni correction for multiple comparisons (alpha* < 0.0007).

DISCUSSION

In this study of longitudinal changes in bronchial inflammatory mediators following smoke inhalation in burn victims, there were marked significant increases in concentrations of IL-1β and IL-8 within six hours of intubation and an increase in percent neutrophils within 12 hours of smoke exposure. These data are consistent with the expectation that early increases in proinflammatory cytokines precede development of clinical lung injury. To our knowledge there are no previously published longitudinal studies of inflammatory cytokines specifically in smoke inhalation victims, although these mediators have been reported to increase in ARDS, in cross-sectional analysis of smoke inhalation victims or in animal studies of smoke exposure.

IL-1ß is a pro-inflammatory cytokine, secreted by epithelial cells, macrophages and monocytes, which increases recruitment of neutrophils and other inflammatory cells and stimulates the secretion of other pro-inflammatory cytokines, and is a major contributor to pro-inflammatory activity in ARDS (Pugin et al., 1996). IL-1β has been found in bronchoalveolar lavage (BAL) fluid at the onset of ARDS, and is spontaneously released from alveolar macrophages (Jacobs et al., 1989). IL-1β levels in patients with ARDS for 7 days are directly correlated with survival (Goodman et al., 1996). Intratracheal delivery of IL-1ß to rats results in a rapid development of pulmonary neutrophilia and neutrophil-dependent increased lung permeability with edema formation (Hybertson et al., 1997). In the normal physiological state, IL-1ß activity is counter-regulated by the naturally occurring interleukin-1 receptor antagonist and by a circulating IL-1 receptor (Martin 1999). The increase in IL-1β reported here is consistent with neutrophil recruitment following smoke inhalation, and may thus help to initiate pathophysiological changes.

IL-8 is a pro-inflammatory cytokine produced by epithelial cells and macrophages and known to play a role in recruiting neutrophils. Neutrophilic activity in BAL is thought to be primarily due to IL-8, though there appears to be poor correlation between IL-8 and PMN at the onset of ARDS (Martin 1999). Levels of IL-8 are elevated in bronchoalveolar lavage (BAL) fluid of ARDS patients (Aggarwal et al., 2000), although the concentration of anti-IL-8:IL-8 complexes may be a better predictor of development of ARDS (Kurdowska et al., 2001). In patients with ARDS, plasma concentrations of IL-8 on day 1 were predictors of subsequent mortality, and plasma concentrations of IL-8 increased over time (Amat et al., 2000). Similarly, high concentrations of anti-IL-8:IL-8 complexes on day 1 were associated with increased risk of ARDS and death (Kurdowska et al., 2001). In a rabbit model, intravenous treatment with a monoclonal anti-IL-8 antibody prevented smoke-induced changes in alveolar epithelium and vascular endothelium (Laffon et al., 1999).

Contrary to expectations, initial tracheobronchial IL-8 concentrations were lower in subjects who later developed more severe lung injury as measured by minimum PaO2/FIO2 ratio with PEEP <6. A similar trend (non-significant) was observed with IL-1β, which is not surprising given the high correlation between these two cytokines. One potential explanation includes modifications to or loss of IL-8 production in patients with more severe smoke exposures. The rise in IL-8 could also be accompanied by an even greater rise in IL-8 antagonists (Kurdowska et al., 2001**),** which might overcome the effect of rising IL-8 concentrations. In future studies it will be important to measure anti-IL-8 concentrations to determine the predicted ability of the IL-8:anti IL-8 ratio for subsequent extent of lung injury. Given the recognized pro-inflammatory activity of IL-8, our finding

of an apparent initial protective effect of this cytokine needs to be validated in future studies.

TNF-α concentrations in the tracheobronchal fluid samples of smoke inhalation victims in our study showed a non-significant increase over time since exposure. TNF-α is thought to directly mediate transcapillary fluid leakage (Shanley et al., 1997) and to promote the adherence of neutrophils to endothelial cells (Gamble et al, 1985). Although TNF-α levels in BAL fluid of ARDS patients have been shown to be elevated in the early phases of ARDS (Tran Van Nhieu et al., 1993; Bhatia and Moochhala, 2004), BAL fluid and pulmonary edema fluid levels of TNF-α have not been consistently correlated with clinical outcomes in ALI (Park et al, 2001; Parsons et al., 2005; Hyers et al., 1991; Pittet et al., 2001). In addition, lymph accumulation of TNF-α was not found to be involved in smoke-induced ALI in sheep (Hales et al., 1997). However, antibodies to TNF-α have been shown to limit septic shock-associated ARDS (Tracey et al., 1987). In addition, reduction in macrophage production of TNF-α through treatment with a free radical scavenger was associated with a marked improvement in smoke-induced lung injury in a rabbit model (Wang et al., 1997).

In cross-sectional analyses, increased TGF-β1 concentrations were associated with improved oxygenation, although none of these findings were statistically significant when adjusting for multiple comparisons. Our findings of a potential protective effect of TGF-β1 in smoke inhalation are somewhat in contrast with the adverse effects of more chronic increases in TGF-β1. TGF-β1 is produced by both epithelial cells and inflammatory cells, including macrophages. Its role in chronic lung inflammation as an inducer of fibrosis has been described in several studies (Broekelmann et al 1991; Border et al 1992). Murine studies indicate an

early increase in TGF-β1 in response to bleomycin-induced ALI/ARDS, and a role in

increasing permeability of pulmonary epithelial cells mediated via integrin pathways (Munger

et al., 1999; Pittet et al., 2001). Increased TGF-β1 expression has been correlated with COPD

and asthma and may be involved in misrepair of the epithelial layer in these chronic

obstructive lung diseases (de Boer et al. 1998; Chung and Barnes 1999; Holgate 2000; Chung

2001). *In vitro* studies demonstrated the effect of TGF-β1 in increasing the permeability of

endothelial monocellular layers (Hurst et al., 1999). TGF-β1 has also been shown to induce

production of procollagen-III by fibroblasts, which has been described as one of the significant

and sensitive predictors of grave prognosis of ALI (Chestnutt 1997).

The other results of our study vary in terms of agreement with previous studies of smoke

inhalation injury. Whereas we found that the patients that died had higher scores on the

bronchoscopic index than did survivors, another study found that the bronchoscopic index

correlated poorly with the level of PEEP required to maintain oxygenation and did not

correlate with duration of intubation in survivors (Bingham et al., 1987). C5a and sFasL have

not been studied in association with smoke inhalation injury. However, C5a and sFasL were

detected in the bronchial lavage fluid of ARDS patients and the concentration of sFasL at the

onset of ARDS was significantly higher in patients who died (Robbins et al., 1987; Matute-

Bello et al., 1999). For IL-10, no studies of ARDS in smoke inhalation victims were found,

but sputum IL-10 did decrease with low-level smoke exposure (Burgess et al., 2002). For

ARDS from all causes, plasma IL-10 levels were increased in at-risk patients but did not

predict the development of ARDS, and initial IL-10 levels were significantly higher in patients

who died compared with survivors (Parsons et al., 1997). However, IL-10 concentrations in

the plasma and BAL fluid of patients with ARDS were significantly lower and the TNF-α/IL-

10 ratio in BAL fluid was significantly higher than in at risk patients (Armstrong and Millar, 1997). No difference in total protein concentration and neutrophil counts was found comparing at-risk patients developing and not developing ARDS (Kurdowska et al., 2001).

Some of the disagreements discussed above may be due to limitations inherent to our study. The number of subjects was relatively small, and information on the extent of smoke exposure was not available. We were not able to rule out cardiac failure in all patients, so strict adherence to ALI and ARDS criteria was not possible. Furthermore, ABGs and chest x-rays were taken based on clinical needs, rather than research needs, so precise determination of time to development of ALI or ARDS was not possible. We did not evaluate outcome past 72 hours, except for mortality. Since blood samples were not available, it was not possible to determine the relationship between tracheobronchial and blood levels of the inflammatory mediators, and the two combined might have greater predictive value. Given the severity of smoke inhalation and generally high percentage of total body surface area burned, the results of this study may not be generalizable to smoke inhalation patients treated outside of a burn center. Although use of DTT to homogenize sputum samples is standard protocol, DTT may interfere with some inflammatory mediator measurements (Woolhouse et al., 2002). However, since all samples were treated in a similar fashion, this potential for interference should be non-differential in nature, and other researchers have not reported an effect on levels of IL-8 and IL-1β in sputum samples (Bhowmik et al., 2000; Stockley et al., 2000; Woolhouse et al., 2002). We also did not look at mediator activity levels or the ratio of cytokines with their inhibitors, such as IL-1β/IL-1RA and IL-8/anti-IL-8 (Park et al., 2001; Kurdowska et al., 2001), which might provide better measures of overall pro- or anti-inflammatory effect.

In conclusion, tracheobronchial secretions are easily collected in intubated smoke

inhalation victims and may have prognostic value in determining the extent of subsequent lung

injury. As expected, there was a marked temporal increase in concentrations of the pro-

inflammatory cytokines IL-1β and IL-8 following smoke exposure. Although initial

concentrations of IL-8 were statistically higher in subjects developing less severe lung injury,

this finding needs to be validated in future studies, in conjunction with measurement of IL-8

inhibitors. Analysis of additional subjects is also needed to further explore the preliminary

cross-sectional findings in the current study suggesting a possible protective effect of TGF- β1.

REFERENCES

Aggarwal A, Baker CS, Evans TW, Haslam PL. G-CSF and IL-8 but not GM-CSF correlate with severity of pulmonary neutrophilia in acute respiratory distress syndrome. *Eur Respir J* 2000;15(5):895-901.

Amat M, Barcons M, Mancebo J, Mateo J, Oliver A, Mayoral JF, Fontcuberta J, Vila L. Evolution of leukotriene B4, peptide leukotrienes, and interleukin-8 plasma concentrations in patients at risk of acute respiratory distress syndrome and with acute respiratory distress syndrome: mortality prognostic study. *Crit Care Med* 2000;28(1):57-62.

Armstrong L, Millar AB. Relative production of tumour necrosis factor alpha and interleukin 10 in adult respiratory distress syndrome. *Thorax* 1997;52(5):442-446.

Border WA, Noble NA, Yamamoto T, Harper JR, Yamaguchi Y, Pierschbacher MD, Ruoslahti E. Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. *Nature* 1992;360(6402):361-364.

Bhowmik A, Seemungal TAR, Sapsford RJ, Wedzicha, JA. Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. *Thorax* 2000; 55:114-120.

Bingham HG, Gallagher TJ, Powell MD. Early bronchoscopy as a predictor of ventilatory support for burned patients. *Journal of Trauma-Injury Infection & Critical Care* 1987;27(11):1286-1288.

Brigham PA, McLoughlin E. Burn incidence and medical care use in the United States: estimates, trends, and data sources. *J Burn Care Rehabil* 1996;17(2):95-107.

Broekelmann TJ, Limper AH, Colby TV, McDonald JA. Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci USA* 1991;88(15):6642-6646.

Burgess JL, Nanson CJ, Hysong TA, Gerkin R, Witten ML, Lantz RC. Rapid decline in sputum IL-10 concentration following occupational smoke exposure. *Inhalation Toxicology* 2002;14(2):133-140.

Chestnutt AN, Matthay MA, Tibayan FA, Clark JG. Early detection of type III procollagen peptide in acute lung injury. Pathogenetic and prognostic significance. *Am J Respir Crit Care Med* 1997;156:840-845.

Chung KF, Barnes PJ. Cytokines in asthma. *Thorax* 1999;54:825-857.

Chung KF. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2001;34:50s-59s.

de Boer WI, van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, van Krieken JH. Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;158:1951-1957.

Enkhbaatar P, Traber DL. Pathophysiology of acute lung injury in combined burn and smoke inhalation injury. *Clin Sci* 2004;107:137-143.

Gamble JR, Harlan JM, Klebanoff SJ, Vadas MA. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc Natl Acad Sci USA* 1985;82(24):8667-8671.

Goodman RB, Pugin J, Lee JS, Matthay MA. Cytokine-mediated inflammation in acute lung injury. *Cytokine Growth Factor Rev* 2003;14:523-535.

Goodman RB, Strieter RM, Martin DP, Steinberg KP, Milberg JA, Maunder RJ, Kunkel SL, Walz A, Hudson LD, Martin TR. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1996; 154:602-611.

Hales CA, Elsasser TH, Ocampo P, Efimova O. TNF-α in smoke inhalation lung injury. *J Appl Physiol* 1997;82(5):1433-1437.

Hales CA, Musto S, Hutchison WG, Mahoney E. BW-755C diminishes smoke-induced pulmonary edema*. J Appl Physiol* 1995;78(1):64-69.

Holgate ST. Epithelial damage and response. *Clin Exp Allergy* 2000;30 (Suppl 1):37-41.

Hurst V, Goldberg PL, Minnear FL, Heimark RL, Vincent PA. Rearrangement of adherens junctions by transforming growth factor-beta1: role of contraction. *Am J Physiol* 1999;276:L582-L595.

Hybertson BM, Lee YM, Repine JE. Phagocytes and acute lung injury: dual roles for interleukin-1. *Ann N Y Acad Sci* 1997;832:266-273.

Hyers TM, Tricomi SM, Dettenmeier PA, Fowler AA. Tumor necrosis factor levels in serum and bronchoalveolar lavage fluid of patients with the adult respiratory distress syndrome. *Am Rev Respir Dis* 1991 Aug;144(2):268-271.

Jacobs RF, Tabor DR, Burks AW, et al. Elevated interleukin-1 release by human alveolar macrophages during the adult respiratory distress syndrome. *Am Rev Respir Dis* 1989;140:1686-1692.

Kiehl MG, Ostermann H, Thomas M, Muller C, Cassens U, Kienast J. Inflammatory mediators in bronchoalveolar lavage fluid and plasma in leukocytopenic patients with septic shock-induced acute respiratory distress syndrome. *Crit Care Med* 1998;26(7):1194-1199.

Kurdowska A, Noble JM, Steinberg KP, Ruzinski JT, Hudson LD, Martin TR. Anti-interleukin 8 autoantibody: interleukin 8 complexes in the acute respiratory distress syndrome. Relationship between the complexes and clinical disease activity. *Am J Respir Crit Care Med* 2001;163(2):463-468.

Laffon M, Pittet JF, Modelska K, Matthay MA, Young DM. Interleukin-8 mediates injury from smoke inhalation to both the lung endothelial and the alveolar epithelial barriers in rabbits. *Am J Respir Crit Care Med* 1999;160(5 Pt 1):1443-1449.

LaLonde C, Ikegami K, Demling R. Aerosolized deferoxamine prevents lung and systemic injury caused by smoke inhalation. *J Appl Physiol* 1994;77(5):2057-2064.

Lewandowski K. Epidemiological data challenge ARDS/ALI definition. *Intensive Care Med* 1999 Sep;25(9):884-886.

Martin TR. Lung cytokines and ARDS. *Chest* 1999;116:2S-8S.

Martin TR, Hagimoto N, Nakamura M, Matute-Bello G. Apoptosis and epithelial injury in the lungs. *Proc Am Thoracic Soc* 2005;2:214-220.

Matthay MA, Zimmerman GA. Acute lung injury and the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2005;33:319-327.

Matute-Bello G, Liles WC, Steinberg KP, Kiener PA, Mongovin S, Chi EY, Jonas M, Martin TR. Soluble Fas ligand induces epithelial cell apoptosis in humans with acute lung injury (ARDS). *J Immunol* 1999;163(4):2217-2225.

Modelska K, Pittet JF, Folkesson HG, Courtney-Broaddus V, Matthay MA. Acid-induced lung injury. Protective effect of anti-interleukin-8 pretreatment on alveolar epithelial barrier function in rabbits. *Am J Respir Crit Care Med* 1999;160(5 Pt 1):1450-1456.

Moore BB, Paine III R, Christensen PJ, Moore TA, Sitterding S, Ngan R, Wilke CA, Kuziel WA, Toews GB. Protection from pulmonary fibrosis in the absence of CCR2 signaling. *J Immuno* 2001;167:4368-4377.

Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 1999;96(3):319-328.

Park WY, Goodman RB, Steinberg KP, Ruzinski JT, Radella II F, Park DR, Pugin J, Skerrett SJ, Hudson LD, Martin TR. Cytokine balance in the lungs of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2001;164:1896-1903.

Parsons PE, Moss M, Vannice JL, Moore EE, Moore FA, Repine JE. Circulating IL-1ra and IL-10 levels are increased but do not predict the development of acute respiratory distress syndrome in at-risk patients. *Am J Respir Crit Care Med* 1997;155(4):1469-1473.

Parsons PE, Moore FA, Moore EE, Ikle DN, Henson PM, Worthen GS. Studies on the role of tumor necrosis factor in adult respiratory distress syndrome. *Am Rev Respir Dis* 1992;146(3):694-700.

Pittet JF, Griffiths MJ, Geiser T, Kaminski N, Dalton SL, Huang X, Brown LA, Gotwals PJ, Koteliansky VE, Matthay MA, et al. TGF-{beta} is a critical mediator of acute lung injury. *J Clin Invest* 2001;107:1537-1544.

Pugin J, Ricou B, Steinberg KP, Suter PM, Martin TR. Proinflammatory activity in bronchoalveolar lavage fluids from patients with ARDS, a prominent role for interleukin-1. *Am J Respir Crit Care Med* 1996;153(6 Pt 1):1850-1856.

Robbins RA, Russ WD, Rasmussen JK, Clayton MM. Activation of the complement system in the adult respiratory distress syndrome. *Am Rev Respir Dis* 1987;135(3):651-658.

Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD. Incidence and outcomes of acute lung injury. *NEJM* 2005;353:1685-1693.

Shanley TP, Foreback JL, Remick DG, Ulich TR, Kunkel SL, Ward PA. Regulatory effects of interleukin-6 in immunoglobulin G immune-complex-induced lung injury. *Am J Pathol* 1997 Jul;151(1):193-203.

Stata 9.1 for Windows. StatCorp LP, College Station, TX. 2005.

Stockley RA, Bayley DL. Validation of assays for inflammatory mediators in sputum. *Eur Respir J* 2000;15:778-781.

Suter PM, Suter S, Girardin E, Roux-Lombard P, Grau GE, Dayer JM. High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon, and elastase, in patients with adult respiratory distress syndrome after trauma, shock, or sepsis. *Am Rev Respir Dis* 1992;145(5):1016-1022.

Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey TJ 3rd, Zentella A, Albert JD, et al. Shock and tissue injury induced by recombinant human cachectin. *Science* 1986;234(4775):470-474.

Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* 1987;330(6149):662-664.

Tran Van Nhieu J, Misset B, Lebargy F, Carlet J, Bernaudin JF. Expression of tumor necrosis factor-alpha gene in alveolar macrophages from patients with the adult respiratory distress syndrome. *Am Rev Respir Dis* 1993;147:1585-1589.

Wang S, Lantz RC, Chen GJ, Breceda V, Rider E, Hays AM, Parliman G, Tollinger BJ, Robledo RF, Kunke K, et al. The prophylactic effects of U75412E pretreatment in a smoke-induced lung injury rabbit model. *Pharmacol Toxicol* 1996;79:231-237.

Wang S, Lantz RC, Rider ED, Chen GJ, Breceda V, Hays AM, Robledo RF, Tollinger BJ, Dinesh SV, Witten ML. A free radical scavenger (Lazaroid U75412E) attenuates tumor necrosis factor-alpha generation in a rabbit model of smoke-induced lung injury. *Respiration* 1997;64:358-363.

Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334-1349.

Woolhouse IS, Bayley DL, Stockley RA. Effect of sputum processing with dithiothreitol on the detection of inflammatory mediators in chronic bronchitis and bronchiectasis. *Thorax* 2002;57:667-671.

Table 1: Characteristics of consented subjects

Mean age (range)	39.2 yrs (1.8 - 88)
Male	51/61 (83.6%)
Total body surface burn	
<15%	23/61 (37.7%)
15-35%	17/61 (27.9%)
>35%	21/61 (34.4%)
Bronchial severity	
1	14/51 (27.5%)
2	16/51 (31.4%)
3	11/51 (21.6%)
4	7/51 (13.7%)
5	3/51 (5.9%)
Organ failure	31/60 (51.7%)
Chest infiltrates*	13/23 (56.5%)
Trauma	9/60 (15.0%)
Fracture	6/60 (10.0%)
$PaO_2/FIO_2 \leq 200$ at ≤ 72 hrs	38/58 (65.5%)
Died within 72 hrs	3/61 (4.9%)

* Only evaluated in subjects with measured tracheobronchial mediator concentrations collected within 6 hours of intubation.

Table 2. Comparison of burn subject characteristics by different outcome measures within 72 hrs of exposure: PaO2/FIO2 ≤ 200 v. > 200; PaO2/FIO2 ≤ 200 v. > 200 restricted to PEEP < 6; and presence v. absence of ARDS (bilateral infiltrates and PaO2/FIO2 ≤ 200).

N (%)	PaO_2/FIO_2 ≤ 200 (n=38)	PaO_2/FIO_2 > 200 (n=20)	PaO_2/FIO_2 ≤ 200 & PEEP<6 (n=21)	PaO_2/FIO_2 > 200& PEEP<6 (n=20)	ARDS (n=8)	not ARDS (n=15)
Males	34 (89.5)	15 (75)	19 (90.5)	14 (70)	8 (100)	14 (93.3)
Females	4 (10.5)	5 (25)	2 (9.5)	6 (30)	0	1 (6.7)
Total burn						
<15%	11 (28.9)	11 (55)	8 (38.1)	10 (50)	0	8 (53.3)
15-35%	10 (26.3)	6 (30)	4 (19.0)	7 (35)	1 (12.5)	3 (20.0)
>35%	17 (44.7)	3 (15)*	9 (42.9)	3 (15)	7 (87.5)	4 (26.7)**
Organ failure	20 (54.1)	9 (45)	9 (45)	7 (35)	4 (50)	5 (33.3)
Trauma	6 (16.2)	3 (15)	4 (50)	1 (5)	3 (37.5)	2 (13.3)
Fracture	4 (10.8)	2 (10)	2 (10)	1 (5)	2 (25)	1 (6.7)

Fisher's Exact test, * p=0.048, ** p=0.011

Table 3. Median, sample size (n), and interquartile range of initial concentrations of

inflammatory biomarkers (within six hours of intubation) by different outcome measures

within 72 hrs of exposure: PaO2/FIO2 ≤ 200 v. > 200; PaO2/FIO2 ≤ 200 v. > 200 restricted

to PEEP < 6; and presence v. absence of ARDS (bilateral infiltrates and PaO2/FIO2 ≤ 200).

	$PaO_2/FIO_2 \leq$ 200	$PaO_2/FIO_2 >$ 200	$PaO_2/FIO_2 \leq$ 200 & PEEP<6	$PaO_2/FIO_2 >$ 200& PEEP<6	ARDS	not ARDS
IL-1β (pg/ml)	822 (n=20)	1630 (n=10)	359 (n=12)	1991 (n=0)	870 (n=8)	676 (n=15)
	(215, 2054)	(214, 4296)	(189,1344)	(456,5469)	(251,7591)	(185, 2567)
IL-8 (pg/ml)	11590 (n=20)	26902 (n=9)	9007[†] (n=12)	23954[†] (n=8)	11998 (n=8)	11491 (n=14)
	(2055, 26506)	(7284, 61001)	(1592, 16968)	(11539, 73676)	(4546, 60083)	(1743, 36265)
IL-10 (pg/ml)	3.02 (n=14)	8.6 (n=6)	2.9 (n=11)	5.25 (n=6)	3.8 (n=8)	3.1 (n=12)
	(0.5, 5.79)	(26.1, 652)	(0.5, 4.47)	(0.5, 15)	(0.5, 6.79)	(0.5, 13.0)
TNF-α (pg/ml)	68.3 (n=20)	179 (n=9)	50.4 (n=12)	186 (n=8)	70.2 (n=8)	75.8 (n=14)
	(17.8, 209)	(26.1, 652)	(14.7, 172)	(47.9, 213)	(8.3, 328)	(16.6, 430)
TGF-β1 (pg/ml)	66.5 (n=14)	98.8 (n=7)	26.6 (n=11)	98.8 (n=7)	117 (n=7)	60.6 (n=14)
	(0.8, 242)	(11.4, 411)	(9.8, 233)	(11.4, 200)	(9.8, 233)	(9.8, 270)
C5a (pg/ml)	1.96 (n=6)	2.8 (n=4)	0.06 (n=5)	2.78 (n=4)	0.053[‡] (n=2)	3.8[‡] (n=8)
	(0.05, 11.3)	(0.5, 9.2)	(0.05, 12.8)	(0.5, 9.2)	(0.05, 0.056)	(0.51, 10.2)
sFasL (pg/ml)	1.01(n=15)	10.2 (n=8)	1.01 (n=11)	10.2 (n=8)	25.0 (n=8)	1.2 (n=15)
	(1.01, 34.6)	(1.1, 42.9)	(1.01, 34.6)	(1.0, 39.7)	(1.0, 36.1)	(1.0, 34.0)

[†] p = 0.030, T-test on log-transformed values (2-sided), unequal variances assumed

‡ p = 0.044, Mann-Whitney U test (exact, 2-sided)

Figure 1. PaO$_2$/FIO$_2$ ratios are inversely related to time since exposure (p=0.001 in a random

coefficients model).

Figure 2. Mean cytokine levels in tracheobronchial fluid samples over time since intubation. A)

Mean levels of IL-8, IL-1β, and TNF-α.   B) Mean levels of TGF-β1, sFasL, IL-10 and C5a.

Figure 1
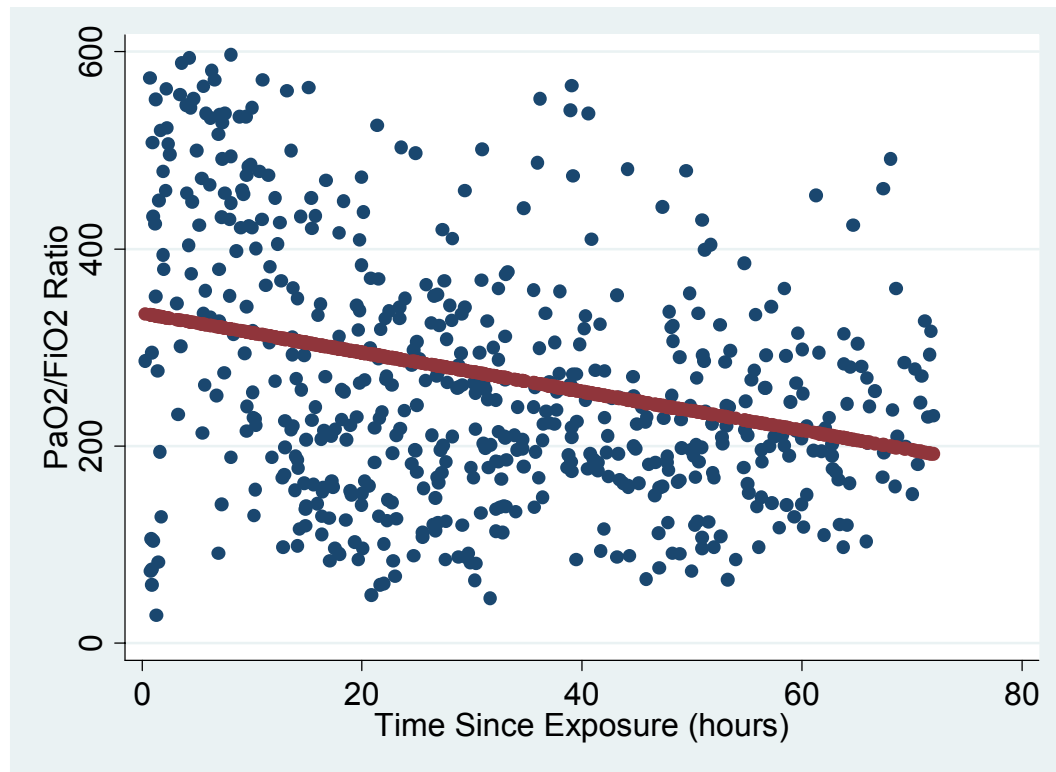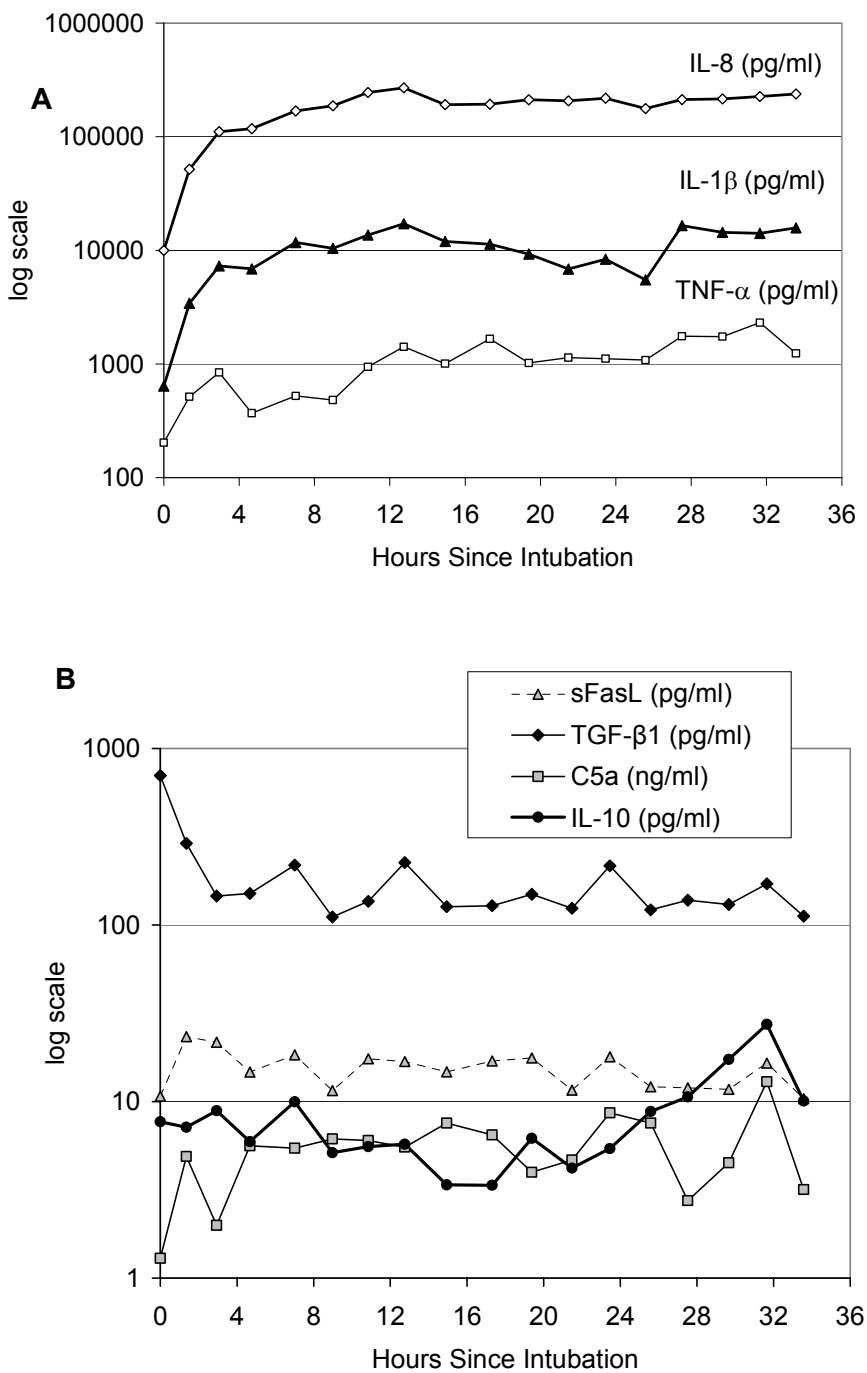
Figure 2.

Sample Collection and Processing

Introduction:
The study is intended to understand the process of respiratory injury in smoke inhalation victims. Looking for early indicators of Acute Lung Injury (ALI) / Adult Respiratory Distress Syndrome (ARDS) is one of them. In general all the patients who are intubated for any medical reasons related to smoke inhalation injury are the study subjects. Tracheo-bronchial suction materials are collected from all the candidates and processed for Interleukins. These materials are collected every 2 hours until the patient recovers from the injury or worsens to ARDS. Maximum time allowed for sample collection is 72 hours. Every week these specimens are brought back from Maricopa Medical Center to University of Arizona and analytical assays are performed for IL-8, IL-10, TNF-alpha, and substance -P concentrations in the processed sample. Duration of study is 3 years.

Study personnel

Name	Role
Dr. Jeff Burgess, M.D	***Principal Investigator***
Dr. Kevin Foster, M.D	Co- Principal Investigator
Dr. Duane Sherrill, PhD	Statistician
Sandra Duran	**Graduate Research Assistant**
Mark	Respiratory Therapist
Don	Respiratory Therapist
Peggy Chrisman	Graduate Research Assistant
Satyarth Kulshrestha	Graduate Research Assistant
Arun Josyula	Graduate Research Assistant

Specimen Collection Procedure

1. *Type of patient*

All Arizona Burn Center patients who are intubated for any medical reason

2. *Specimen collection*

i. Connect Lukens-Trap and suction tube to endo-tracheal tube.
ii. Inject specified amount of NS in to patient's trachea through ET tube.
iii. Use the following chart to find out how much of NS to be injected based on patients' age.
iv. Then aspirate as much content as possible.
v. Transfer the aspirate to a new 50-ml conical vial.
vi. If the sample is thick, use a new scoop to transfer the content.

	Age	.ml of NS to be injected
1	Younger than 2 years	5 ml
2	2 – 15 years	10 ml
3	Older than 15 years	15 ml

3. *Saving sample in methanol("Methanol")*

i. Using a new scoop each time, transfer scoop full of aspirate (up to the brim) into a 50ml conical vial labeled "M" (for methanol), containing 6 ml of 100% Methanol.
ii. If the aspirate is thick or sticky, use another new scoop to push it in to conical vial containing methanol.
iii. After transferring the contents to vial, Mix it thoroughly and close the cap tightly.
iv. Use the styro-form cases to keep them up right in the refrigerator.
v. Label it and store it in refrigerator (NOT FREEZER).
vi. Please mention any problems associated with any of these sample in the sample collection form.

4. *Saving remaining sample("Frozen")*

i. After the methanol sample is saved, close the cap tightly, label appropriately and stick a cello tape over the label to avoid loosening from freezing.
ii. Use styro-form cases to keep these tubes upright in the freezer.
iii. Store it in freezer at -80 C.
iv. Fill the sample collection form with initial and time.

If you have any questions, ask Sandra Duran or Arun at (520)-405-2716.

Transportation

Stocked specimens are transported to U of A every 1-2 weeks. (Arun)

Processing "frozen" sample for collection of supernatant

i. Allow the sample to thaw to room temperature, using a beaker of warm water if needed.
ii. Note the serial number and the volume of each sample in the "supernatant tracking form".
iii. For each sample add 4.5 ml of PBS and 0.5 cc of Sputolysin.
iv. Vortex the sample for I 0 min to mix the contents thoroughly. Use extra time if the sample is too thick.
v. Spin the sample in centrifuge at 2000 RPM for 20 min.
vi. Note the amount of supernatant and cell pellet in ml and enter in the "supernatant tracking form".

> If the amount if supernatant is less than 5 ml, centrifuge the sample again at 2000 RPM for 20 min. Note the action in the "supernatant tracking form".

vii. Transfer all the supernatant into five 2 ml cryo-vials and remaining supernatant into 5 ml cryo-vial.
viii. Label and store them in freezer at -80 C.
ix. Discard remaining contents.

Processing "methanol" preserved sample

i. Allow the sample to reach room temperature.
ii. Note the volume of the sample in the "methanol sample tracking form"
iii. Add required amount of PBS to make the total volume of 9 ml and then add 1 ml of Sputolysin.(ask Dr. Burgess, and Peggy)(supposed dilution of 1:5)

> DONOT ADD SPUTOLYCIN TO COLD SAMPLE AND DONOT ADD IT BEFORE ADDING PBS.

iv. Mix the contents thoroughly using vortex for about 10 min for sputolysin to act.
v. Add required amount of PBS to the remaining contents to make total volume of 20ml. (final dilution of 1:10)
vi. Note the volumes in the "methanol sample tracking form".
vii. Mix thoroughly using vortex to make the solution uniform.
viii. Save solution for cell counts and cyto-spins for differential counts (go to next section for cell counts using this sample).

Cell-count technique

i. Mix the contents by hand shaking.

ii. Using a new pipette tip each time, take 10 μl of the solution prepared in the above step and transfer the contents to a small 2ml conical vial.(blue coloured)

iii. Take 90 μl of tryptan blue and add it to the sample in small conical vial. (this gives a dilution of 1:10 and a final dilution of 1: 100)

iv. Mix the contents thoroughly using the same pipette tip.

v. Label it and proceed to next step of counting cells

vi. Mount 10 μl of the "tryptan blue added sample" on hemocytometer chamber.

vii. Count white cells in 4 large corner squares(16 small squares in each corner square) in hemocytometer

{If cell distribution is too uneven or if you see a large mucus plug discard it and make a fresh dilution and repeat the process (from i to vii)}

viii. Note down the total count in "methanol sample tracking form".

Calculations

For the above-mentioned dilutions i.e. 1:100, use the following formula.
Total number of cells in 20 ml of solution = [Total count/4] X 10^6
Cell concentration (# / ml)= total cells / 2 ml (volume of undiluted sample)

Differential counts

❑ **Making the solution of desired concentration of cells**

i. After obtaining the cell count; use the following formula to make a solution with a cell concentration of one million cells per ml.

Use C1.V1 = C2.V2 for calculating the amount of PBS to be added, where C1 is concentration in the 20 ml solution prepared (find out by dividing the actual concentration by 10), **V1 is the volume of that solution to be taken**, C2 is 1million / ml and V2 is 2 ml.

---

ii. After calculating the volume V1, take that amount in to a 2 cc cryo vial and add PBS to make it 2 ml9find out the volume by subtracting V1 from 2 ml, [volume of PBS to be added = 2 – V1])

iii. Label for identification and mix the sample thoroughly with the same pipette.

❑ **Spin process**

i.   Label two cyto-spin slides for each sample and place them with filter paper and conical spin funnel in the "cyto-spin centrifuge"
ii.   Using a micro pipette, Add 50 µL of thawed bovine serum in the funnel.
iii. Using a new tip each time, add 50 µL of the thoroughly mixed, diluted sample in the funnel
iv.   Close the lid and spin the machine at 800-1000 RPM for 5-6 min
v.   After the centrifuge stops take the lid off and remove the slides carefully without disrupting relative position of the filter paper on the slide
vi.   Keep the slides face up and let air dry

❑ **Staining**

i.   Pour the staining solutions in respective white staining containers
ii.   Stain each slide by dipping 5 times for one second each time (in order) in fixative soln, soln 1, soln 2 and finally rinse in distilled water.
iii. Place them on a piece of paper and let air-dry.
iv.   Once the slides are dry they are ready for microscopy. (differentials)

❑ **Counting and storing**

i.   Count differential and enter the values in "differential counts form"
ii.   Save all labeled slides in slide box.

UNIVERSITY OF ARIZONA HUMAN SUBJECTS PROTECTION PROGRAM
Verification of Human Subjects Training

Title of Project: ACUTE LUNG INJURY FOLLOWING SMOKE INHALATION: PREDICTIVE VALUE OF SPUTUM BIOMARKERS AND TIME COURSE OF LUNG INFLAMMATION

All individuals conducting research involving human subjects (with or without financial support of any sponsoring organization or agency) must complete Human Subjects training. Those individuals include principal investigators, co-investigators and all other individuals involved in the conduct of research. Students and their advisors must meet the same standard as faculty and staff.

I hereby certify that individuals involved in this proposal have completed the required Human Subjects training.

_____	_____3/29/06_____
Principal Investigator's Signature	Date

Please list all individuals involved in the above-cited research study

Name	Research Role (PI, Co-PI, Collaborator, Sub-I, Data Manager, Research Assistant, etc.)	Will this person be involved in the consenting process? *	Training Title Indicate type of training: **Med-I**, **Med-II**, and/or **SBS** (see definitions below)**	Completion Date(s) for each Human Subjects training listed (mm/dd/yy)
Jeff Burgess	PI	YES NO	Med-I	8/17/01
Kevin Foster	Co-PI	YES NO	Med-I	3/21/02
Arun Josyula	Research Assistant	YES NO	Med-I	4/11/03
Sally Littau	Laboratory Coordinator	YES NO	Med-I	3/08/04
Margaret Kurzius Spencer	Research Specialist	YES NO	Med-I	7/03/01
Kamiar Massrour	Radiologist	YES NO	Med-I	10/28/03
Simon Wong	Investigator	YES NO	Med-I	2/17/06

*Consent forms are to be signed and dated by the subject (or their legal representative) and by the Principal Investigator or Co-Principal Investigator (no other study personnel may sign as Investigator without prior approval of the IRB). Other study personnel involved in the consenting process may sign as Presenter, but not as Investigator.

DUE DATE: 1 APRIL 2005	HUMAN SUBJECTS COMMITTEE PERIODIC REVIEW FORM	APPROVAL EXPIRES: 4/15/2005

**Jefferey Burgess/A02.18/Publ Hlth/Acute Lung Injury Following Smoke Inhalation: Predictive Value
of Sputum Biomarkers and Time Course of Lung Inflammation**
NAME OF INVESTIGATOR/PROJECT APPROVAL NUMBER/TITLE OF PROPOSAL

Human subjects approval for this activity expires on the date indicated above. Depending upon the activity status of the project, attachments may be required. Refer to IRB website (www.irb.arizona.edu) for detailed instructions. **Note:** If renewal is not granted before the expiration date, all study activities must stop at that time. If study procedures/treatment must be continued for subject safety, contact the IRB office immediately.

Activity Status – check one box only
Category A: attach items 1-13 listed on reverse
- ☑ Enrollment of new subjects in progress
- ❏ Enrollment not initiated, but still planned
- ❏ Enrollment closed to new subjects but current subjects are still undergoing study procedure or being entered into extensions and/or sub-studies

Category B: attach items 1-12 listed on reverse
- ❏ Enrollment closed, follow-up only (**non-sensitive** data collection via telephone contact, questionnaire and/or record review)
- ❏ Local data analysis only: no subject contact/no additional data collection (annual review required)

Category C: attach items 1-8 listed on reverse
- ❏ Concluded: enrollment and all participation/follow-up/local data analysis completed

Category D: no attachments required; complete and submit this form only
- ❏ Study not begun: permanent withdrawal of study

Subject Numbers (local enrollment)
If more than one study population is involved, report enrollment under number 2 of checklist (see reverse)
a) Number of new subjects enrolled (consented) since last reporting period _____ 49
b) Total number of subjects enrolled (consented) since start of project _____ 61
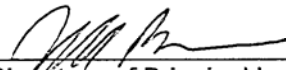c) Male/female ratio of total enrolled since start of project _____ 51/10

Conflict of Interest Statement (COI): see COI policies at http://vpr2.admin.arizona.edu/rie/conflilct_of_interest.htm
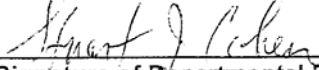a) Do any of the investigators serve as a speaker or consultant to the sponsor, the manufacturer, or the owner of the test article? ❏ Yes ☑ No
b) Do any of the investigators (or their family members) derive a direct or indirect benefit equity and/or royalty relationship with the sponsor, manufacturer, or owner of the test article? ❏ Yes ☑ No
If yes to either of the above, **attach** copy of U of A Conflict of Interest and Commitment Disclosure form.

I certify that this research will be conducted in accordance with the currently approved protocol/amendments and that no changes to procedures or study documents will be made without the knowledge/approval of the IRB.

_____	3-21-05	_____	3-25-05
Signature of Principal Investigator (required for all projects)	Date	**Signature of Departmental Review Chair** (not required for concluded or not begun studies)	Date

---

FOR COMMITTEE USE ONLY

☒ Approve ❏ Disapprove   Period of Approval: APR 2 5 2005 ___ APR 1 5 2006

Subject to the following conditions: PI to submit complete copy of grant proposal for IRB review/
records. Collaborating site re-approval/reapproved consent instruments to be submitted to
UA IRB as soon as available (current approval expires 5/5/05). UA IRB approval stamp has
been placed on current consent version approved for use - does not
need to be duplicated for use with subjects). Personnel changes (removing Hysong, Francis,
                                                              and Bojorquez; adding
_____   Date Reviewed: APR 2 5 2005 Kurzius Spencer) approv-
David G. Johnson, M.D., Chair   concurrently.
Biomedical/Continuing Review Committee   ☒ Expedited Review ❏ Full Committee Review
Lapse in approval due to delay in IRB processing; existing subjects allowed to continue.

March 29, 2006

David G. Johnson, MD
Chairman
Human Subjects Committee
1350 N. Vine Avenue
P.O. Box 245137
Tucson, AZ 85724-6721

Re: HSC A 02.18 ACUTE LUNG INJURY FOLLOWING SMOKE INHALATION: PREDICTIVE VALUE
OF SPUTUM BIOMARKERS AND TIME COURSE OF LUNG INFLAMMATION

Dear Dr. Johnson:

For the continuing review of this project, the following information and attachments are submitted.

1) Consent forms (MIHS, not U. of Arizona): a) Subject's Consent Form, b) Parental Consent Form, c) Child's
   Assent Form, and d) Adolescent Assent Form.
2) Summary of progress to date: We have continued to enroll subjects. We are continuing to perform analyses
   on concentrations of inflammatory mediators in the tracheobronchal suctionate material.
3) The only change since the last program approval was the addition of a consulting radiologist Dr. Kamiar
   Massrour.
4) There were no adverse events/unanticipated problems to local subjects or study personnel since the last
   approval.
5) There is no information in the medical literature that I am aware of that would change the risk/benefit ratio or
   willingness of a subject to participate in the study.
6) No subjects have withdrawn from the study.
7) No complaints have been received.
8) A copy of a recently submitted manuscript is attached. We are in the progress of writing up this year's report
   to the sponsor but have not completed it yet.
9) The abstract on the attached manuscript describes the study well.
10) This study continues to be supported by the U.S. Army Peer Review Medical Research Program.
11) Consent forms are provided by MIHS.
12) A current VOTF is attached.
13) There is no information in the medical literature that I am aware of that would change the description of the
    risks, benefits or procedures described on the consent form.

Sincerely,

Jefferey L. Burgess, MD, MPH